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The CD4-Mediated Immune Response Is Critical in Determining the Outcome of Infection Using Theiler's Viruses with VP1 Capsid Protein Point Mutations

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Daniel's strain of Theiler's virus (DA) induces a chronic demyelinating disease in the central nervous system (CNS) of susceptible SJL mice, which serves as an excellent model of multiple sclerosis. We previously demonstrated that point mutations near a putative virus receptor-binding site [VP1 99 (Gly to Ser) or 100 (Gly to Asp)] totally attenuate the ability of DA to persist and induce demyelination in SJL mice. The current studies demonstrate that class II-restricted $CD4^+$ T cells play a major role in clearing VP1 mutant DA viruses from the CNS to prevent demyelination. Infection of SJL $CD4^{(-/-)}$ mice with DA-VP1-99(Ser) or DA-VP1-100(Asp) resulted in virus persistence and prominent demyelination in the spinal cord. In contrast, infection of SJL $CD8^{(-/-)}$ mice with DA-VP1-99(Ser) or DA-VP1-100 did not result in virus persistence or demyelination. In addition, no virus-specific cytotoxicity was observed in CNS-infiltrating lymphocytes following infection of SJL mice with VP1 mutant viruses. The mutant DA-VP1-99(Ser) and DA-VP1(100) viruses were in fact neurovirulent when compared to the wild-type DA virus, as they induced an overwhelming encephalitis and early lethality (2 to 4 days postinfection) in mice deficient in the IFN-α/β receptor. Therefore, the nondemyelinating phenotype observed with DA-VP1-99(Ser) and DA-VP1-100(Asp) viruses is dependent in part on the CD4-mediated host immune response.

INTRODUCTION

The Daniel strain (DA) of Theiler's murine encephalomyelitis virus (TMEV) induces chronic inflammatory demyelination and virus persistence in the spinal cord of susceptible strains of mice, but is cleared from the central nervous system (CNS) of resistant strains of mice (Clatch *et al.,* 1985; Rodriguez *et al.,* 1986). The mechanisms by which this potentially lytic virus establishes a persistent infection and induces demyelination in the CNS of susceptible mice are not completely understood. However, the immune system has been shown to have a significant role in resistance to persistent infection as well as to susceptibility to TMEV-induced demyelination. TMEV-induced demyelinating disease is immune-mediated (Lipton and Dal Canto, 1976) and provides an excellent viral model for human multiple sclerosis.

A major goal in TMEV biology has been to identify the molecular components of the viral genome that predispose mice to viral persistence and demyelination. A variant of wild-type DA (wt-DA), designated as DA-P12, was isolated during the 12th passage of a persistently infected G26-20 glioma cell line (Patick *et al.,* 1990). DA-P12 failed to persist and induce demyelination in susceptible SJL/J mice. Molecular sequencing of DA-P12 showed that three point mutations in VP1 [residues 99(Gly to Ser), 100(Gly to Asp), and 103(Asn to Lys)] (Lin *et al.,* 1998) had occurred during the G26-20 glioma cell passage. Using wt-DA and DA-P12 recombinant and mutant virus studies, we showed that mutations at VP1 residues 99 or 100, but not at 103, were responsible for the inability of DA-P12 to induce demyelination and persist in SJL mice. Because the mutants grew less well than wt-DA in the CNS of B6×129 $RAG1^{-/-}$ mice deficient in T and B cells, the change in the CNS disease phenotype of the mutant viruses was related in part to decreased neurovirulence. However, the failure to induce demyelination and persist was not due to a general inability of these mutant viruses to grow, since they replicated in L-2 cells *in vitro* as effectively as wt-DA. We proposed originally that a disruption of the interaction between the mutant DA viruses and the receptors on certain neural cells *in vivo* might have altered the ability of the mutant viruses to demyelinate. However, the difference in CNS growth was not a complete explanation for the failure of mutant DA-VP1-99(Ser) and DA-VP1-100(Asp) to demyelinate, since DA-VP1-103(Lys) virus was able to induce demyelination and persist in SJL mice despite having a similar decrease in neurovirulence in B6×129 $RAG1^{-/-}$ mice. We therefore considered that

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TABLE 1

Pathologic Scores in the Spinal Cords of SJL, SJL CD4$^{-/-}$, and SJL CD8$^{-/-}$ Mice 45 Days following Infection with Mutant or DAFL3 Viruses

			% Quadrants (mean ± SEM) positive for abnormality		
Virus	Mouse strain	*N***	Gray matter inflammation	White matter inflammation	Demyelination
DA-VP1-99(Ser)	SJL	10	0.0 ± 0.0	0.2 ± 0.2*	0.0 ± 0.0*
DA-VP1-100(Asp)	SJL	5	0.0 ± 0.0	0.0 ± 0.0*	0.0 ± 0.0*
DA-VP1-103(Lys)	SJL	6	0.0 ± 0.0	22.8 ± 8.5	17.9 ± 8.0
DAFL3	SJL	15	0.2 ± 0.2	26.3 ± 5.6	24.9 ± 5.5
DA-VP1-99(Ser)	SJL CD4$^{-/-}$	4	0.0 ± 0.0	6.0 ± 2.4	21.7 ± 7.8*
DA-VP1-100(Asp)	SJL CD4$^{-/-}$	4	0.0 ± 0.0	7.3 ± 3.7	13.6 ± 7.1*
DA-VP1-103(Lys)	SJL CD4$^{-/-}$	5	1.0 ± 0.7	20.1 ± 6.3	43.1 ± 12.1*
DAFL3	SJL CD4$^{-/-}$	5	0.4 ± 0.4	23.7 ± 4.3	71.9 ± 7.9
DA-VP1-99(Ser)	SJL CD8$^{-/-}$	6	0.0 ± 0.0	1.1 ± 0.8	0.7 ± 0.4
DA-VP1-100(Asp)	SJL CD8$^{-/-}$	6	0.0 ± 0.0	0.3 ± 0.3*	0.0 ± 0.0*
DA-VP1-103(Lys)	SJL CD8$^{-/-}$	5	0.0 ± 0.0	2.5 ± 1.8	2.9 ± 2.1
DAFL3	SJL CD8$^{-/-}$	4	0.0 ± 0.0	15.2 ± 3.9	12.7 ± 2.4

* Represents a statistical difference from DAFL3 within an individual strain of mice ($P < 0.05$). Statistical differences were detected using a one-way ANOVA on ranks. Pair-wise comparisons were performed using Dunn's method.

** N = number of mice.

other mechanisms, possibly immunologic, might also be involved in the altered phenotype observed following infection with the mutant viruses.

The immune response is a critical factor in the control of Theiler's virus infection in the CNS (Rodriguez *et al.,* 1991). Both class I-restricted CD8$^+$ T cells and class II-restricted CD4$^+$ T cells contribute to virus clearance and to resistance to virus-induced demyelination (Lin *et al.,* 1997, 1999; Murray *et al.,* 1998; Nicholson *et al.,* 1996; Njenga *et al.,* 1996; Rivera-Quinones *et al.,* 1998; Rodriguez *et al.,* 1991). In mice of a resistant H-2^b genotype, depletion of T cell subsets with monoclonal antibodies directed against CD4$^+$ or CD8$^+$ T cells results in an increased frequency of paralysis and death due to acute encephalitis (Rodriguez *et al.,* 1991). Genetic deficiency of class II-mediated immune responses or CD4$^+$ T cells (Murray *et al.,* 1998; Njenga *et al.,* 1996) or deficiency of class I-mediated responses or CD8$^+$ T cells (Murray *et al.,* 1998; Rivera-Quinones *et al.,* 1998) predisposes mice of a normally resistant H-2^b haplotype to DA-induced demyelination. In mice of susceptible SJL background, genetic deletion of CD4$^+$ T cells results in severe demyelination, profound neurologic dysfunction, and increased virus load following infection with wild-type virus (Murray *et al.,* 1998). In contrast, genetic deletion of CD8$^+$ T cells does not significantly alter the extent of demyelination compared to wt-DA infection of SJL mice (Murray *et al.,* 1998). These experiments implicate a critical role for CD4$^+$ T cells in control of virus replication and protection from demyelinating disease in mice of susceptible genotype. These experiments also indicate that in mice of a susceptible genotype, CD8 T cells play a minimal role in the control of virus replication.

Even though both CD4$^+$ and CD8$^+$ T cells play an important role in protecting resistant mice from demyelination and persistence, there are other factors that appear to have an even greater role in protecting mice from acute lethal encephalitis. The most important of these is IFN-α/β. IFN-α/β is important in preventing death and controlling acute TMEV infection. Resistant haplotype H-2^b mice deficient in IFN-α/β receptors die of overwhelming encephalitis within a few days of infection (Fiette *et al.,* 1995; Njenga *et al.,* 1997). IFN-α/β has a direct role in controlling virus spread and also appears to be a principal mediator of MHC class I expression in the CNS following TMEV infection (Njenga *et al.,* 1997; Van den Broek *et al.,* 1995).

In the present study, we evaluated the phenotype of TMEV-induced disease following infection with mutant viruses DA-VP1-99(Ser), DA-VP1-100(Asp), and DA-VP1-103(Lys) in susceptible SJL mice with a genetic deficiency of CD4$^+$ T cells or CD8$^+$ T cells. We also evaluated the neurovirulence of the mutant viruses by infecting mice deficient in IFN-α/β receptors. The results indicate that the class II-restricted CD4$^+$ T cell-mediated immune response rather than the class I-restricted CD8$^+$ T cell response contributes to the nondemyelinating phenotype of mutant attenuated DA-VP1-99(Ser) and DA-VP1-100(Asp) viruses in normally susceptible SJL mice.

RESULTS

Mutant DA viruses induce demyelination in the spinal cord of SJL CD4$^{-/-}$ mice but not in that of SJL CD8$^{-/-}$ mice

We showed previously that mutant DA-VP1-99(Ser) and DA-VP1-100(Asp) viruses failed to induce demyelination in the spinal cord of highly susceptible SJL mice (Lin *et al.,* 1998). This was confirmed in the present study when mice infected with DA-VP1-99(Ser) and DA-VP1-100(Asp)

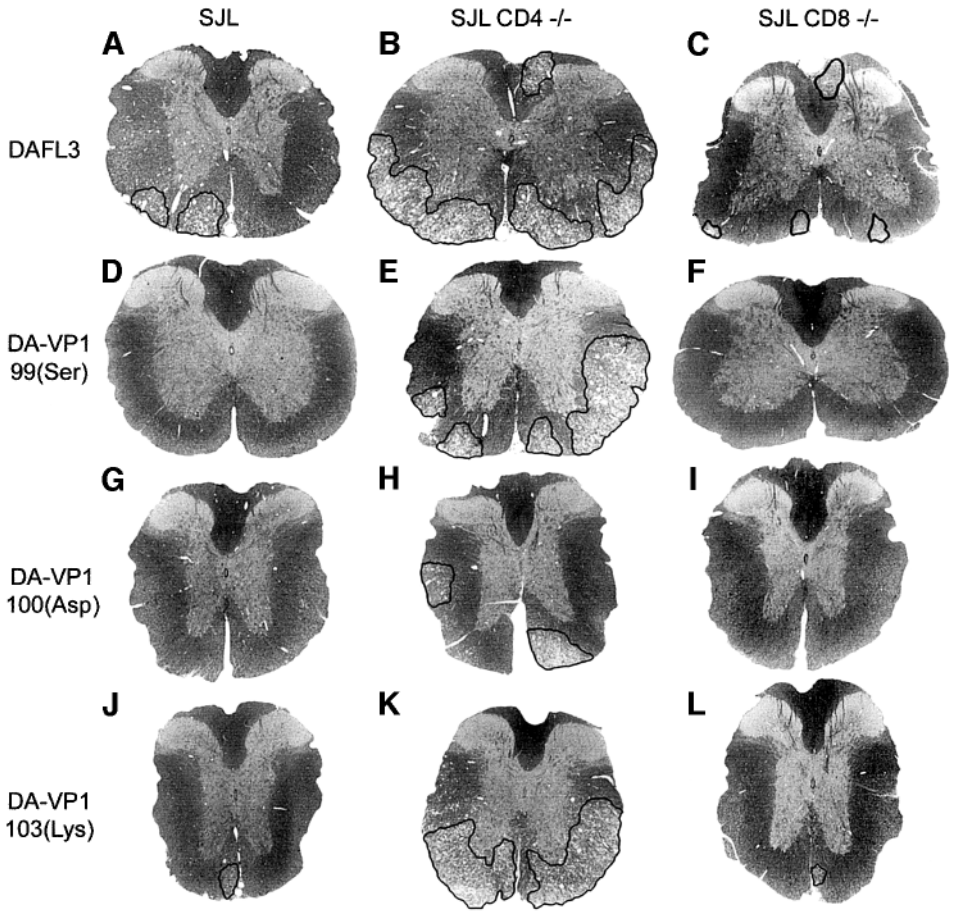

FIG. 1. Mutant DA and DAFL3 viruses induce chronic demyelination and white matter inflammation in the CNS of SJL CD4$^{-/-}$ mice. SJL mice (A, D, G, J), SJL CD4$^{-/-}$ mice (B, E, H, K), and SJL CD8$^{-/-}$ mice (C, F, I, L) were infected with DAFL3 (A, B, C), DA-VP1-99(Ser) (D, E, F), DA-VP1-100(Asp) (G, H, I), or DA-VP1-103(Lys) (J, K, L) viruses. DAFL3 (A) and DA-VP1-103(Lys) (J) induced demyelination (black outline) in immunocompetent SJL mice, whereas DA-VP1-99 (D) and DA-VP-100 (G) did not. DAFL3 (B), DA-VP1-99(Ser) (E), DA-VP1-100(Asp) (H), and DA-VP1-103(Lys) (K) viruses induced demyelination and white matter inflammation in the spinal cords of SJL/J CD4$^{-/-}$ mice. In contrast, no demyelination was observed in spinal cords of SJL CD8$^{-/-}$ mice infected with DA-VP1-99(Ser) (F) or DA-VP1-100(Asp) (I). Small demyelinating lesions were observed in SJL CD8$^{-/-}$ mice infected with DA-VP1-100(Lys) (L) and DAFL3 (C). Magnification, ×400. Plastic-embedded sections were stained with a modified erichrome/cresyl violet stain. Quantitation of the number of quadrants showing pathology is shown in Table 1.

were examined at 45 days postinfection (d.p.i.) (Table 1, Fig. 1). The 45-day time point was chosen because this has been proven to be a reliable time for distinguishing virus persistence and demyelination in animals of susceptible versus resistant genotypes (Rodriguez *et al.,* 1986). In contrast, infection with DA-VP1-103(Lys) and wild-type DAFL3 resulted in prominent demyelination in the spinal cord at 45 d.p.i. (Table 1, Fig. 1). Previous studies have demonstrated that CD4$^+$ T cells are not necessary for TMEV-induced demyelination because severe white matter lesions are observed in the CNS of SJL CD4$^{-/-}$ mice infected with DAFL3 (Murray *et al.,* 1998). In the present study, we tested whether the absence of demyelination in SJL mice following infection with DA-VP1-99(Ser) and DA-VP1-100(Asp) was due to clearance by a CD4$^+$ or CD8$^+$ T cell-mediated immune response. We found prominent demyelination in SJL CD4$^{-/-}$ mice infected with each of the mutant viruses (Table 1, Fig. 1), suggesting that the lack of demyelination observed following infection with DA-VP1-99(Ser) or DA-VP1-100(Asp) virus was explained partly by protection from a CD4$^+$ T cell-mediated immune response. In SJL CD4$^{-/-}$ mice, DA-VP1-99(Ser) and DA-VP1-100(Asp) viruses induced significantly less demyelination and white matter inflammation in the spinal cords than infection with mutant DA-VP1-103(Lys) and DAFL3 ($P < 0.05$) (Table 1, Fig. 1). Interestingly, minimal or no demyelination was observed in the spinal cords of SJL CD8$^{-/-}$ mice infected with mutant DA-VP1-99(Ser), DA-VP1-100(Asp), or DA-VP1-103(Lys) virus. In contrast, demyelination was observed in the spinal cords of SJL CD8$^{-/-}$ mice infected with DAFL3; however, this pathology was reduced compared to DAFL3 infection of wild-type SJL mice. At present, the factors contributing to this reduced pathology are unknown. In general, more demyelination was observed following DAFL3 or VP1 mutant virus infection in SJL CD4$^{-/-}$ mice than in SJL CD8$^{-/-}$ or wild-type SJL mice. These results are consistent with the hypothesis that a

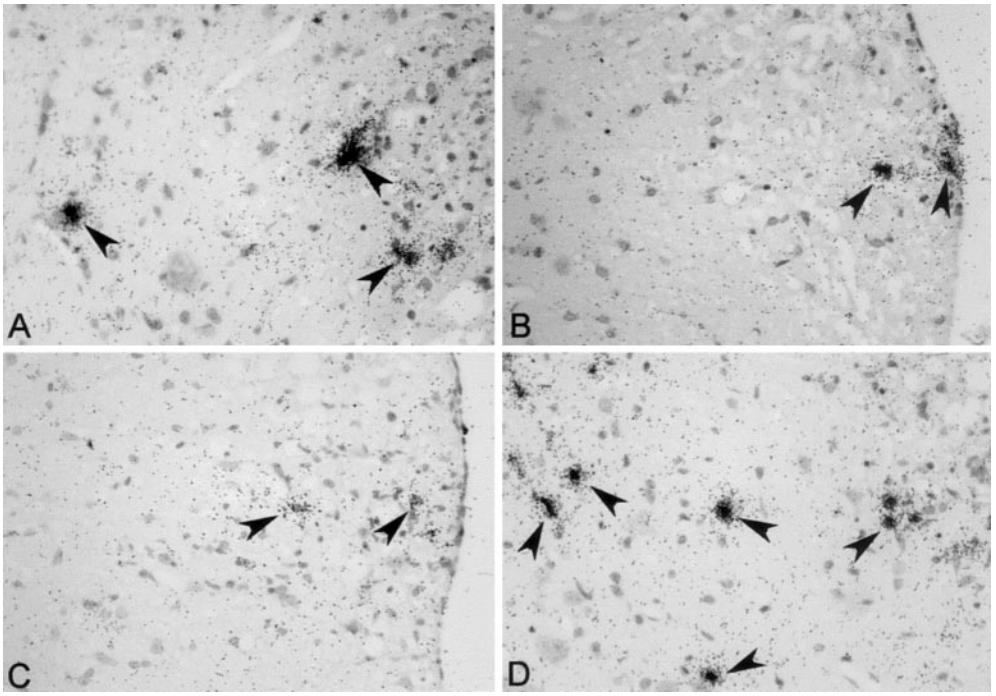

FIG. 2. *In situ* hybridization was used to detect virus genome in the spinal cords of SJL CD4$^{-/-}$ mice infected with (A) DAFL3, (B) DA-VP1-99(Ser), (C) DA-VP1-100(Asp), and (D) DA-VP1-103(Lys). Arrows show virus-RNA-positive cells localized exclusively in the white matter. See text for quantitation of virus-RNA-positive cells/mm^2 of spinal cord. Similar experiments in SJL CD8$^{-/-}$ mice showed no virus-RNA-positive cells in mice infected with DA-VP1-99(Ser) or DA-VP1-100(Asp). Only rarely were positive cells observed following infection with DA-VP1-103(Lys). In contrast, cells frequently were found following infection with DAFL3.

CD4$^+$ T cell-mediated immune response plays a critical role in protection from demyelination following infection with either the wild-type or the VP1 mutant virus. Therefore, the presence or absence of the CD4-mediated immune response determines whether demyelination develops following infection with these mutant viruses. However, the severity of pathology may be determined by additional factors, such as virus–cell receptor interactions (Lin *et al.,* 1998).

Mutant DA viruses persist in the CNS of susceptible genotype (SJL) mice deficient in CD4$^+$ T cells

To test whether the mutant viruses persist in the spinal cords of SJL mice deficient in CD4$^+$ or CD8$^+$ T cells, we used *in situ* hybridization with a ^{35}S-labeled VP2 probe to localize DAFL3 and VP1 mutant viral RNA in mice infected for 45 days (Fig. 2). This allowed us to precisely relate the extent of demyelination to the level of virus RNA persistence in the same animals. Virus RNA was demonstrated in the spinal cord white matter but not in the gray matter of SJL CD4$^{-/-}$ mice infected with all three mutant and DAFL3 viruses [virus-positive cells/mm^2 were 4.4 ± 2.0 (DA-VP1-99-Ser), 1.3 ± 0.8 (DA-VP1-100-Asp), 10.9 ± 5.5 (DA-VP1-103-Lys), and 15.0 ± 6.5 (DAFL3)]. Of interest, virus-positive cells/mm^2 in SJL CD4$^{-/-}$ mice infected with DA-VP1-99(Ser) and DA-VP1-100(Asp) were fewer in number than those in mice infected with DA-VP1-103(Lys) or DAFL3. However, the results did not reach statistical significance. No virus-RNA-positive cells were identified in the spinal cords of SJL CD8$^{-/-}$ mice infected with DA-VP1-99(Ser) and DA-VP1-100(Asp). A low level of virus RNA was demonstrated in the spinal cord of one of three SJL CD8$^{-/-}$ mice infected with DA-VP1-103(Lys), which showed small demyelinating lesions. In contrast, viral RNA was demonstrated in all three SJL CD8$^{-/-}$ mice infected with DAFL3. These results indicated that the presence of virus genome in the spinal cord of mice infected with DAFL3 or mutant viruses coincided with the development of demyelination. In addition, the severity of demyelination (Table 1) correlated almost perfectly ($r = 0.98$, $P = 0.02$) with the number of virus-RNA-positive cells in the spinal cords of SJL CD4$^{-/-}$ mice. Therefore, CD4$^+$ T cells, but not CD8$^+$ T cells, play a critical role in the clearance of mutant DA-VP1-99(Ser) and DA-VP1-100(Asp) viruses from the CNS of normally susceptible SJL mice.

To confirm this conclusion, we assessed whether deletion of CD4$^+$ T cells would predispose mice to infectious virus persistence as detected by plaque assay. SJL and SJL CD4$^{-/-}$ mice were sacrificed 45 d.p.i., and the CNS homogenates were prepared for plaque assays to determine the levels of infectious virus. Infectious DA-VP1-99(Ser) and DA-VP1-100(Asp) persisted in the CNS of SJL CD4$^{-/-}$ mice, although the titers were significantly lower ($P < 0.05$) than those of mice infected with DAFL3 and DA-VP1-103(Lys) viruses (Table 2). In contrast, no

TABLE 2

Virus Titers in the CNS of SJL and SJL CD4$^{-/-}$ Mice Infected with DAFL3 or Mutant DA Viruses

Mouse strain	DA-VP1-99(Ser)	DA-VP1-100(Asp)	DA-VP1-103(Lys)	DAFL3
SJL	0.00 ± 0.00**	0.00 ± 0.00**	3.14 ± 0.89	1.89 ± 0.54
SJL CD4$^{-/-}$	2.51 ± 0.45*	2.14 ± 1.16*	5.17 ± 0.28	5.36 ± 0.40

Note. Brain and spinal cords were isolated from mice 45 days following intracerebral inoculation with DAFL3 or mutant DA viruses, homogenized, and sonicated for plaque assay. Virus titers are expressed as mean ± standard error ($\log_{10}$ PFU/g CNS tissue). The sensitivity of plaque assay was 1.7 $\log_{10}$ PFU/g CNS tissue. Student's *t* test was used to compare virus titers in each mouse strain infected with mutant viruses with mice infected with DAFL3.

* $P < 0.05$.

** $P < 0.01$.

infectious DA-VP1-99(Ser) or DA-VP1-100(Asp) was detected in the CNS of immunocompetent SJL mice (Table 2). These studies supported the hypothesis that a CD4$^+$ T cell immune response contributes to the clearance of the mutant viruses from the CNS of susceptible SJL mice. Plaque assays were not done for SJL CD8$^{-/-}$ mice because the pathologic studies demonstrated no demyelination or viral RNA by *in situ* hybridization following infection with DA-VP1-99(Ser) or DA-VP1-100(Asp).

An impaired humoral response is observed in SJL CD4$^{-/-}$ mice infected with VP1 mutant viruses

Having established that the CD4$^+$ T cell immune response contributed to the clearance of DA-VP1-99(Ser) and DA-VP1-100(Asp) in SJL mice, we assessed by ELISA the levels of virus-specific antibodies in the sera of SJL CD4$^{-/-}$ mice compared to those in the sera of immunocompetent SJL and SJL CD8$^{-/-}$ mice. Previous studies have demonstrated that mice deficient in CD4$^+$ cells maintain the ability to isotype switch from IgM to IgG (Rahemtulla *et al.,* 1994). However, it is possible for CD4-deficient mice to have an inefficient humoral response, which may explain the ability of VP1 mutant viruses to persist in the spinal cords of SJL CD4$^{-/-}$ mice. We first assessed the humoral immune responses in immunocompetent SJL, SJL CD8$^{-/-}$, and SJL CD4$^{-/-}$ mice infected with the mutant VP1 viruses versus those in wild-type DAFL3 mice (Figs. 3A to 3D). In general, higher titers of virus-specific antibodies were observed following infection of immunocompetent SJL mice with DA-VP1-99(Ser) and DA-VP1-100(Asp) than in those infected with DA-VP1-103(Lys) and DAFL3. In addition, infection of SJL or SJL CD8$^{-/-}$ mice with DAFL3 or the mutant VP1 virus resulted in higher titers of virus-specific antibody than those observed in SJL CD4$^{-/-}$ mice. These data suggest that a CD4$^+$ T cell-mediated immune response was important in the development of a humoral response against all viruses and that an inefficient humoral response may have contributed to the phenotype observed in SJL CD4$^{-/-}$ mice following infection with DA-VP1-99(Ser) and DA-VP1-100(Asp).

It is also possible that VP1 mutations altered the ability of the humoral response to recognize and neutralize the viruses (Sato *et al.,* 1996). To test this hypothesis, we conducted cross-neutralization experiments to determine whether sera isolated from SJL mice infected with DA-VP1-99(Ser), DA-VP1-100(Asp), and DA-VP1-103(Lys) were capable of neutralizing infectious DAFL3 (Fig. 3E). For a positive control, the results were compared to the sera from SJL mice infected with DAFL3. A plaque assay was performed using sera from DAFL3, DA-VP1-99(Ser)-, DA-VP1-100(Asp)-, and DA-VP1-103(Lys)-infected SJL mice. The $\log_2$ serum dilution required to obtain 50% neutralization of infectious DAFL3 virus (1000 PFU/ml) was significantly greater for the sera collected from SJL mice infected with all viruses than for the sera isolated from uninfected SJL mice ($P < 0.05$). Thus, sera from SJL mice infected with the VP1 mutant viruses can neutralize wild-type DAFL3.

Virus-specific cytotoxicity is not observed in the CNS following infection of SJL mice with VP1 mutant viruses

Previous studies (Lin *et al.,* 1997, 1999) have demonstrated that virus-specific cytotoxic lymphocytes (CTLs) play a critical role in virus clearance from the CNS of resistant H-2^b haplotype mice following wild-type DAFL3 infection. In contrast, susceptible SJL mice mount minimal or no virus-specific CTLs directed against the wild-type DALF3 virus (Lin *et al.,* 1999). Interestingly, a previous study demonstrated virus-specific CTLs in the CNS of SJL mice infected with the mutant DAL*−1 virus (Lin *et al.,* 1999). The mutant DAL*−1 virus fails to synthesize the L* protein and does not persist in the CNS of SJL mice. SJL mice infected with DAL*−1 do have K^s-restricted CTLs in the CNS, suggesting that the generation of a CTL response may be important in the clearance of this virus (Lin *et al.,* 1999). To further investigate the role of CTLs in the clearance of VP1 mutant viruses from the CNS of SJL mice, we assessed whether infection with DA-VP1-99(Ser), DA-VP1-100(Asp), or DA-VP1-103(Lys) would result in a virus-specific CTL response (Table 3). The CNS-infiltrating lymphocytes isolated from SJL mice infected for 7 days with DAFL3 or mutant virus did not

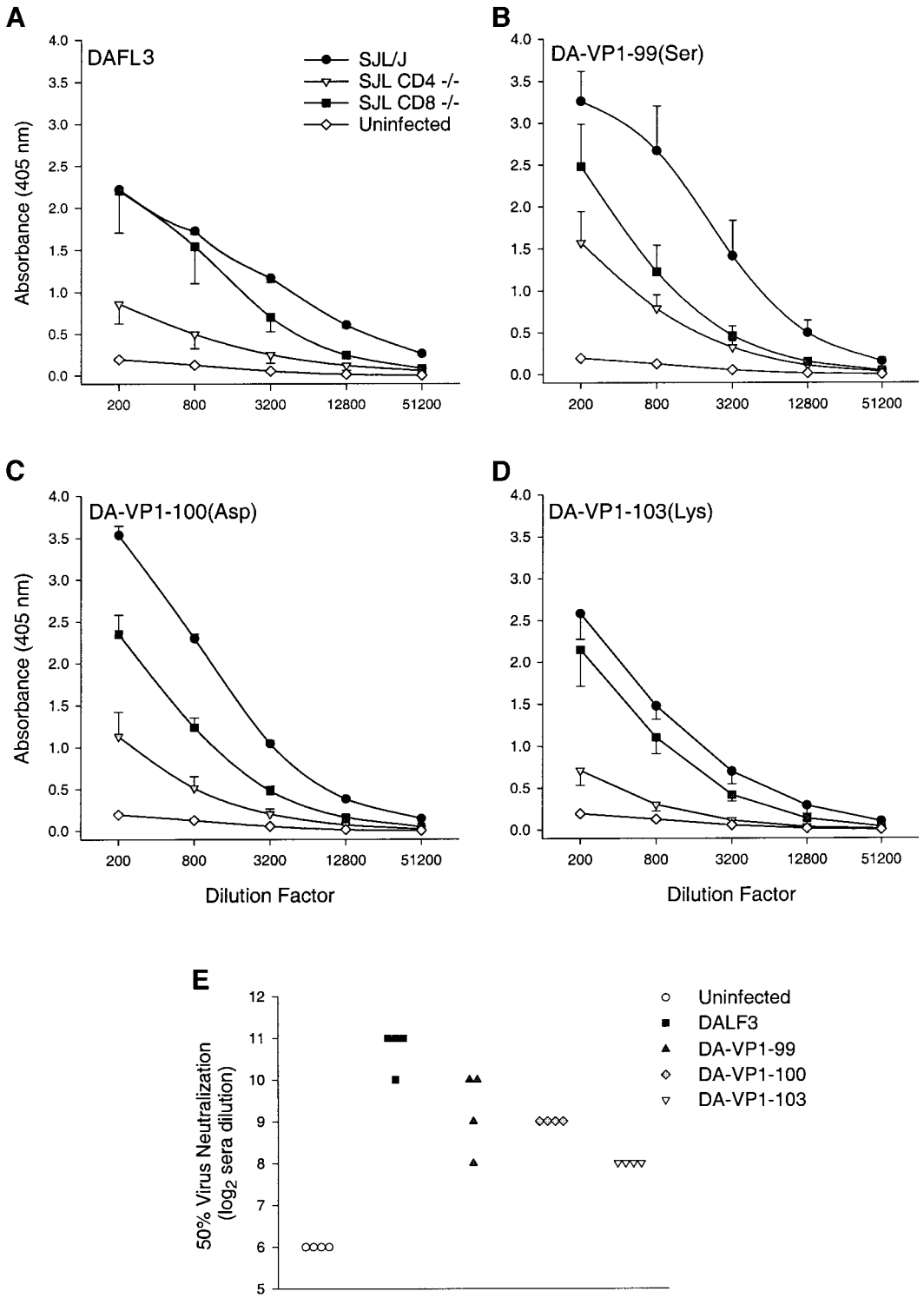

FIG. 3. Characterization of the humoral response to wt-DA antigen following intracerebral infection of SJL, SJL $CD4^{-/-}$ and SJL $CD8^{-/-}$ mice with (A) DAFL3, (B) DA-VP1-99(Ser), (C) DA-VP1-100(Asp) or (D) DA-VP1-103(Lys) viruses. Sera were diluted 1:200 to 1:51,200 in PBS with 0.2% bovine serum albumin. Data are represented as the mean optical density (at 405 nm) ± standard error at each dilution. (E) The $\log_2$ serum dilution required to obtain 50% neutralization of infectious DAFL3 was determined for sera collected from SJL mice infected with DAFL3, DA-VP1-99(Ser), DA-VP1-100(Asp), and DA-VP1-103(Lys). Sera collected from uninfected SJL mice were used as a negative control. Each point on the graph represents serum collected from an individual mouse.

demonstrate virus-specific cytotoxicity, regardless of whether the target cells were infected with the wild-type or mutant virus. In contrast, for positive control, we infected SJL mice with the mutant DAL*−1 virus and demonstrated a CTL response against DAL*−1- or DAFL3-infected target cells. Thus, SJL mice can mount a virus-

TABLE 3

CNS Virus-Specific Cytotoxicity from Infected SJL/J Mice

Virus used to infect SJL/J mice	Virus used to infect target cell line (PSJLSV)	% Specific lysis of target cells (±SEM)
DA-VP1-99(Ser)	Uninfected	0.0 ± 0.0
	DAFL3	0.2 ± 0.2
	DA-VP1-99(Ser)	2.6 ± 1.5
DA-VP1-100(Asp)	Uninfected	0.0 ± 0.0
	DAFL3	0.0 ± 0.0
	DA-VP1-100(Asp)	0.0 ± 0.0
DA-VP1-103(Lys)	Uninfected	0.0 ± 0.1
	DAFL3	0.0 ± 0.0
	DA-VP1-103(Lys)	5.3 ± 0.2
DAFL3	Uninfected	0.0 ± 0.0
	DAFL3	2.0 ± 2.0
	DA-VP1-99(Ser)	1.8 ± 1.8
	DA-VP1-100(Asp)	0.0 ± 0.0
	DA-VP1-103(Lys)	5.1 ± 3.6
DAL*−1	Uninfected	3.3 ± 2.8
	DAFL3	**15.6 ± 2.9**
	DAL*−1	**19.5 ± 8.0**

Note. SJL/J mice were infected intracerebrally with the viruses listed above. CNS-infiltrating lymphocytes from five mice per experimental group were isolated and pooled at 7 days postinfection. PSLSV (K^s, D^s) were used as target cells at an effector to target ratio of 100 to 1. PSLSV target cells were left uninfected (negative control) or were infected with the viruses listed above.

specific CTL response when infected with DAL*−1. These studies provide further support for the hypothesis that $CD8^+$ T cells are not critical for viral clearance and the lack of demyelination observed in SJL mice infected with the VP1 mutant viruses.

The VP1 mutants are neurovirulent, as demonstrated by infection of IFN-α/β-receptor-deficient mice

It was hypothesized previously that disruption of mutant VP1 virus–receptor interactions *in vivo* might have altered the viruses' neurovirulence. To assess the neurovirulence of the mutant viruses, we infected AB129 mice, which are of the resistant haplotype $H\text{-}2^b$ but are deficient in the IFN-α/β receptors. IFN-α/β protect against lethality during the acute phase following Theiler virus infection (Fiette *et al.,* 1995; Njenga *et al.,* 1997; Van den Broek *et al.,* 1995). Infection of AB129 mice with three mutant VP1 viruses or the wild-type DAFL3 virus resulted in death from overwhelming encephalitis at 2 to 4 days postinfection. Abundant virus genome was detected by *in situ* hybridization in the brain and spinal cord of all infected AB129 mice, irrespective of VP1 mutation (data not shown). The results demonstrate that IFN-α/β is critical in protection from overwhelming encephalitis induced by both the mutant and DAFL3 viruses. Thus, the mutant viruses have neurovirulence similar to that of DAFL3 following inoculation of mice deficient in the IFN-α/β receptors. This finding suggests that the mutant DA viruses are not CNS-growth-deficient viruses during early infection, arguing against the simple hypothesis that these viruses are attenuated because they fail to bind to the putative TMEV receptors on susceptible neurons or glial cells.

DISCUSSION

We proposed previously that mutations in DA VP1 residues 99, 100, and 103 disrupt the interaction between TMEV and its receptors on certain CNS cells (Lin *et al.,* 1998), causing a failure of some of the mutant viruses to induce demyelination in susceptible SJL/J mice. In this study we investigated how the immune response was responsible in part for the inability of these mutant viruses to induce demyelination in susceptible SJL/J mice. We utilized SJL $CD4^{-/-}$ mice to test definitively whether class II-restricted $CD4^+$ T cells, which are known to be critical in virus clearance and resistance to TMEV-induced demyelination (Murray *et al.,* 1998; Njenga *et al.,* 1996; Rodriguez *et al.,* 1991), are important in the attenuation of the VP1 mutants. Although we showed that DA-VP1-99(Ser) and DA-VP1-100(Asp) failed to induce demyelination in SJL mice, we demonstrated that these two viruses (as well as DAFL3) persisted in the CNS of SJL $CD4^{-/-}$ mice and induced demyelination in the spinal cord. Of interest, minimal or no demyelination and no virus-RNA-positive cells were observed in the spinal cord of SJL $CD8^{-/-}$ mice infected with DA-VP1-99(Ser) and DA-VP1-100(Asp) mutant viruses. Therefore, the absence of a class II-restricted $CD4^+$ T cell response, but not a class I-restricted $CD8^+$ T cell response, interferes with clearance of both DAFL3 and mutant VP1 viruses. This is consistent with a study by Kurtz and colleagues (1995) that demonstrated the importance of the $CD4^+$ T cell-mediated immune response in viral clearance from SJL mice. The absence of a $CD4^+$ T cell-mediated immune response predisposes normally susceptible SJL mice to enhanced virus persistence and virus-induced demyelination following infection with the VP1 mutant viruses. The results from this study also indicate that the attenuated phenotype observed following the infection of immunocompetent SJL mice with DA-VP1-99(Ser) or DA-VP1-100(Asp) cannot be explained by a disruption in virus–cell receptor interactions per se.

Despite persistent virus replication and subsequent demyelination in $CD4^+$-deficient mice infected with VP1 mutant viruses, some of our data suggest that interference between the virus and its putative receptor on certain CNS cells may also contribute to the phenotype. Virus titers by plaque assay in the CNS of SJL $CD4^{-/-}$ mice infected with mutant DA-VP1-99(Ser) and DA-VP1-100(Asp) were lower than those in mice infected with DAFL3. Thus, mutations in DA VP1 residues 99 and 100 might disrupt the interaction between virus and receptor on certain CNS cells independent of the immune response. A previously described variant of DA virus

(Jnaoui and Michiels, 1998) had mutations in the CD loop of VP1 [aa (amino acid(s)) 100–102] and the EF loop of VP2 (aa 162, 171, and 173) and was attenuated in neurovirulence similar to our mutants following infection of immunocompetent SJL mice. The VP1 amino acid changes in this variant and in our mutant viruses are clustered in a site recognized by neutralizing antibody (Sato *et al.,* 1996; Wada *et al.,* 1994; Zurbriggen *et al.,* 1991) that lies on the rim of a depression in the virion surface called the "pit" (Luo *et al.,* 1992). A mutation at the rim of the pit may interfere with binding of the virus to the receptor of a particular cell type (possibly oligodendrocytes or microglia). It is also possible that the mutations altered the tropism of DA-VP1-99(Ser) and DA-VP1-100(Asp), which has the potential to lessen the severity of demyelination following infection of SJL CD4$^{-/-}$ mice. However, the fact that DA-VP1-99(Ser) and DA-VP1-100(Asp) persisted in the spinal cord white matter of SJL CD4$^{-/-}$ mice and that the percentage of spinal cord demyelination (for all viruses) correlated almost perfectly with the number of virus-positive cells/mm^2 (within the spinal cord white matter) is more consistent with the hypothesis that a reduced ability to interact with a cellular receptor rather than a change in tropism was responsible for the observed phenotype. This hypothesis is also supported by a previous study that demonstrated smaller plaque sizes on L-2 cell monolayers infected with DA-VP1-99(Ser) or DA-VP1-100(Asp) (Lin *et al.,* 1998) than on DAFL3 controls. In any case, future experiments will be designed to definitively prove this hypothesis.

To further assess the neurovirulence of mutant VP1 viruses, we infected mice deficient in the IFN-α/β receptors. IFN-α/β rises rapidly following virus infection, induces a generalized antiviral state in neighboring uninfected cells, and activates macrophages/NK cells (Sen and Lengyel, 1992). IFN-α/β also upregulates MHC class I expression in the CNS of mice following infection with wt-DA virus (Njenga *et al.,* 1997), potentially enhancing cytotoxic lymphocyte function in resistant mice. In this study we demonstrated that the mutant VP1 viruses are neurovirulent and that IFN-α/β plays a critical role in defense against the lethality of both mutant DA and DAFL3 viruses during acute infection. All the viruses had a similar acute lethality following inoculation of AB129 mice deficient in IFN-α/β receptors. The mutant DA-VP1-99(Ser) and DA-VP1-100(Asp) viruses did not manifest their distinctive mutant phenotypes in IFN-α/β receptor knockout mice and behaved similarly to DAFL3 and DA-VP1-103(Lys). Although the two mutant viruses may have decreased virus growth in the CNS compared to DAFL3 (Lin *et al.,* 1998), they are lethal to mice in the face of severe weakening of the immune response that is critical for resistance.

Because a reduction in neurovirulence alone was not responsible for viral clearance and the absence of demyelination observed following infection of immunocompetent SJL mice with DA-VP1-99(Ser) and DA-VP1-100(Asp), we further examined the role of the immune response. The demyelinating phenotype observed following infection of SJL CD4$^{-/-}$ suggested that a CD4$^+$ T cell-mediated immune response was involved. To completely rule out a role for CD8$^+$ T cells in contributing to the attenuated phenotype of the mutant viruses, we assessed virus-specific cytotoxicity in infiltrating lymphocytes from the CNS. This was important because our previous studies with mutant virus DAL*−1 showed that it was likely that the presence of virus-specific CTLs accounted for the absence of virus persistence and demyelination following infection of SJL mice with the mutant DAL*−1 (Lin *et al.,* 1999). Infection of SJL mice with the DAL*−1 virus, which does not synthesize the L* protein, results in the generation of K^s-restricted CTLs in the CNS of SJL mice that appear to be critical for early virus clearance. In contrast to DAL*−1, infection of VP1 mutant viruses did not result in the generation of virus-specific CTLs in the CNS of SJL mice. This fits well with the observation that infection of SJL CD8$^{-/-}$ mice with VP1 mutant viruses did not result in virus persistence or demyelination.

The potential mechanisms by which CD4$^+$ T cells contribute to viral clearance following TMEV infection are multiple and include the optimal generation and maintenance of DA-specific CTLs in the CNS, the secretion of lymphokines that activate either macrophages or NK cell function, the production of virus-specific antibodies, and the direct control of virus infection or amplification (Koszinowski *et al.,* 1991). To address the role of the humoral response, we assessed virus-specific antibody titers in SJL, SJL CD8$^{-/-}$, and SJL CD4$^{-/-}$ mice. Compared to SJL and SJL CD8$^{-/-}$, virus-specific antibody titers were reduced in all SJL CD4$^{-/-}$ mice following infection with both wild-type and mutant viruses. In addition, sera isolated from mice infected with the mutant viruses had reduced ability to neutralize wild-type DAFL3 compared to the sera isolated from mice infected with DAFL3, suggesting that antibodies recognizing the mutant viruses with single point mutations were different from those that recognized the wild-type virus. This may have resulted from the presence of mutations in a major TMEV neutralization site (Sato *et al.,* 1996). Thus, an impaired humoral response in SJL CD4$^{-/-}$ mice may partly explain the demyelinating phenotype observed following infection with DA-VP1-99(Ser) and DA-VP1-100(Asp).

In summary, these results demonstrate the complex mechanisms of the attenuated phenotype and lack of virus persistence/demyelination following infection of highly susceptible SJL mice with VP1 mutant DA viruses. We propose that the DA-VP1-99(Ser) and DA-VP1-100(Asp) viruses may have reduced ability to interact with cellular receptors on CNS cells as a consequence of the VP1 point mutations. This results in a decreased efficiency of infection in immunocompetent SJL mice. Clearance of the mutant viruses involves an intact class

II-restricted immune $CD4^+$ T cell. In the absence of class II-restricted $CD4^+$ T cells, an impaired humoral response may provide these mutant viruses with the opportunity to establish viral persistence in the spinal cord white matter and to induce demyelination. In addition, reduced lymphokine-induced activation of antigen-presenting cells and decreased intracellular antiviral defenses may have also contributed to a broader immunodeficiency that enabled the mutant viruses to persist. Therefore, the ultimate outcome of infection with these VP1 mutant viruses depends on both the host immune defense response and the virulence of the virus.

MATERIALS AND METHODS

TMEV viruses

DAFL3 virus was prepared from a wild-type DA full-length infectious cDNA clone (pDAFL3). Mutant DA-VP1-99(Ser), DA-VP1-100(Asp), and DA-VP1-103(Lys) viruses were generated by site-directed mutagenesis of DAFL3, as previously described (Lin *et al.,* 1998). DAFL3 and the three mutant DA viruses were propagated in BHK-21 cells and used in all the experiments.

Cells

L-2 and BHK-21 cells were cultured in RPMI 1640 (BioWhittaker, Walkersville, MD) with 5% fetal calf serum (GibcoBRL, Grand Island, NY) and used for plaque assays to titrate viruses and for preparation of virus stocks.

Animals

Four- to eight-week-old female SJL/J ($H\text{-}2^s$) and B6 ($H\text{-}2^b$) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Four- to eight-week-old female SJL $CD4^{-/-}$ ($H\text{-}2^s$), SJL $CD8^{-/-}$ ($H\text{-}2^s$), and AB129 ($H\text{-}2^b$) mice deficient in IFN-α/β receptors were bred in the Rodriguez laboratory at the Mayo Clinic. Animals were intracerebrally injected with 2×10^5 PFU of viruses in a 10 μl volume. Care and handling of mice conformed to the guidelines of both the National Institutes of Health and the Mayo Clinic Animal Care and Use Committee.

Pathologic analyses

Mice were anaesthetized with pentobarbital and perfused by intracardiac puncture with Trump's fixative (phosphate-buffered 4% paraformaldehyde and 1% glutaraldehyde, pH 7.2). Spinal cords were removed, sectioned coronally and serially into 15 to 20 blocks, osmicated, and embedded in 2-hydroxyethyl methacrylate (JB-4 system from Polysciences). Sections (2-μm-thick) were stained with a modified erichrome method with cresyl violet counterstain (Pierce and Rodriguez, 1989) to detect demyelination and inflammation. Detailed morphologic analysis was performed by examining each quadrant from 15 to 20 spinal cord coronal sections from each mouse for the presence or absence of demyelination, white matter inflammation, and gray matter inflammation (Rodriguez *et al.,* 1983). The presence or absence of the pathologic abnormality was determined in every spinal cord quadrant. The total score was expressed as the percentage of spinal cord quadrants with the specific abnormality such that a maximum score of 100 represented the presence of disease in every quadrant of every spinal cord section examined. Statistical differences between pathologic scores were assessed using a one-way ANOVA on ranks. Pair-wise comparisons were made using the Dunn method ($P < 0.05$).

Virus plaque assay

Infectious virus titers in the CNS of mice were determined by a plaque assay. Forty-five days postinfection, brains and spinal cords were removed aseptically. CNS homogenates (10%) were prepared in RPMI 1640, sonicated twice for 3 min each, clarified by centrifugation, and stored at −70°C. Virus titers of CNS homogenates were determined by plaque assay on monolayers of L-2 cells, as described previously (Patick *et al.,* 1990). The results were expressed as mean ($\log_{10}$ PFU/g CNS tissue) ± SEM calculated from two to five mice in each group. Statistical differences between groups were detected using an unpaired Student's *t* test ($P < 0.05$).

In situ hybridization for viral RNA

In situ hybridization was used to detect virus genome in the CNS of mice. Sections of paraffin-embedded spinal cord were hybridized overnight with a ^{35}S-labeled probe complementary to the coding region of VP2. Slides were exposed to autoradiography for 48 h after extensive washing, as described previously (Patick *et al.,* 1990). Slides were counterstained with hematoxylin. Data were expressed as the number of virus-RNA-positive cells/mm^2. To calculate this number, we manually counted the virus-RNA-positive cells and divided this by the area of the spinal cord sampled. The area sampled was calculated using a Zeiss interactive digital analysis system (ZIDAS) and a camera lucida attached to a Zeiss photomicroscope. We analyzed from 10.31 mm^2 to 25.79 mm^2 (mean = 18.93 mm^2) of spinal cord from three to four mice per group. Statistical differences were assessed using a one-way ANOVA. Pair-wise comparisons were made using the Student–Newman–Keuls method ($P < 0.05$). The correlation coefficient between spinal cord demyelination and virus-RNA-positive cells/mm^2 in SJL $CD4^{-/-}$ was calculated using a Pearson product moment correlation ($P < 0.05$).

Virus-specific antibody ELISA's

The presence of anti-DA antibodies (IgG + IgM) in the sera of infected mice was determined by an enzyme-linked immunosorbent assay as described previously (Njenga *et al.,* 1996). In brief, polystyrene microtiter plates were coated with purified wild-type DAFL3. Sera

from individual mice (four mice per group) were serially diluted and incubated at room temperature. Biotinylated goat anti-mouse IgG and IgM were used as the secondary antibody. Amplification was performed using alkaline phosphatase conjugated to streptavidin. Detection was performed using *p*-nitrophenyl phosphatase as a substrate. The antibody level was expressed as an optical density reading of 405 nm.

Virus neutralization assay

Samples of TMEV were diluted to contain 50 PFU/0.2 ml and were mixed with an equal volume of two-fold dilutions of heat-inactivated (45 min, 56°C) serum from infected mice or noninfected mice. Four mice were tested per group. After incubation at 25°C for one h, virus–serum mixtures were assayed for infectious virus by plaque assay. Neutralization titers were expressed as the $\log_2$ dilution of serum that resulted in a 50% reduction in virus titer. Statistical differences between $\log_2$ dilutions of serum were detected using a one-way ANOVA. Pair-wise comparisons were made using the Student–Newman–Keuls method ($P < 0.05$).

Virus-specific cytotoxicity assay

SJL/J mice were intracerebrally inoculated with 2×10^5 PFU of mutant viruses, DAFL3, or DAL*−1 virus in a 10-μl volume. Five animals were pooled per experimental group. At 7 d.p.i., CNS-infiltrating lymphocytes were isolated by Percoll gradient as described previously (Lin *et al.,* 1997, 1999) to serve as effectors for the cytotoxicity assay. Uninfected and virus-infected PSJLSV (K^s, D^s) cells were labeled with ^{51}Cr (200 μCi/10^6 cells) for 1 h, washed three times with RPMI 1640, and used as targets for a cytotoxicity assay. The cytotoxicity assay was performed as described previously (Lin *et al.,* 1997, 1999). Mean radioactivity values were calculated from triplicate wells, and results were expressed as the percentage specific lysis according to the formula [(experimental counts − spontaneous counts)/(maximum counts − spontaneous counts)] × 100%. Standard error of the mean was calculated from the pooled lymphocyte samples in triplicate wells.

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